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Wei Liu M.D.
PI - Signature Date

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Specific aims: Insulin-like growth factors (IGFs) have been shown to stimulate cell proliferation and differentiation. And IGF-I, one of the important members of the IGF family plays an important role in the mitogenesis of breast cancer. IGF-I stimulates cell division by modulating events that occur during the early G1 phase. Cell proliferation and activation of oncogenes in cancer cells has been shown to be involved in the modulation of signal transduction pathways. The most important signal transduction pathways in mammalian cells are MAPK pathways, which include ERK1/ERK2, JNK and p38 pathways. These kinases are activated by growth factors and also under stress conditions. Amino acid starvation in cell lines can be used as an experimental model for stress, which can mimic the pathophysiological condition that results from protein deprivation during cancer cachexia. Therefore, we hypothesize that signal transduction pathways in human breast cancer are involved in IGF-I-mediated cellular proliferation under amino acid starvation conditions.

Therefore, the specific aim of this study was designated to test the hypothesis that: The signal transduction pathways in human breast cancer involve activation of MAPK (ERK1/ERK2) and JNK pathways.

Results: We have shown that IGF-I stimulates cyclin D1 expression in human breast cancer cell MCF-7 in a time and dose-dependent manner (**Figure 1**). The maximum stimulation was observed at 18 to 24 hours in the 10 ng/ml of IGF-I concentration. Cyclin D1 mRNA was blocked by the addition of PI-3 kinase inhibitors (**Figure 2**). IGF-I was also shown to mediate the phosphorylation of mitogen-activated protein kinase (ERK1/ERK2) as well as that of protooncogene c-jun in MCF-7 cells (**Figure 3**). Our studies suggest that cyclin D1 expression is regulated by IGF-I at the transcription level and that the phosphorylation pathways are regulated by PI-3 kinase signaling pathway in addition to MAPK pathways.

The effect of amino acid starvation on specific signal pathways was investigated. An increase in phosphorylation of ERK1/ERK2 was observed in a time-dependent manner (**Figure 4**) with peak stimulation after 5 min amino acid withdrawal. Stress-activated protein kinase JNK was also activated in response to amino acid starvation resulting in c-jun phosphorylation (**Figure 5**). In summary, our results indicate those protein kinases; ERK1/ERK2 and JNK play an important role under amino acid starvation-induced stress conditions in MCF-7 cells.

Publications:

1. Liu W, Chillar, and Vadgama JV. IGF-I-induced mitogenesis of breast cancer cells involves in MAPK and the PI-3 kinase pathways. *Pro. Am. Assoc. Cac.* Res. (39):458, 1998.

2. Liu W, Vadgama JV, and Wu Y. Signal transduction pathways in breast cancer cell MCF-7 upon amino acid starvation involved in MAPK and SAPK/JNK pathways. *FASEB J.* (12):A450, 1998.

cc. Mentor: JV. Vadgama, Ph. D.
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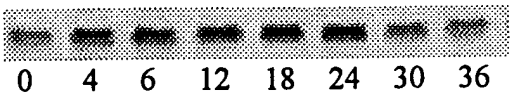
Figure 1.

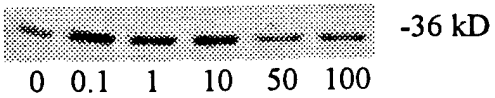
IGF-I Stimulates Cyclin D1 Expression in MCF-7 Cells



Legends:

IGF-I, at 10 ng/ml, upregulates cyclin D1 expression in a time-dependent manner. Maximal regulation was observed after 24 hours, followed with down regulation at 30 and 36 hours.

IGF-I Stimulates Cyclin D1 Expression in MCF-7 Cells: Dose-dependent Test



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This data confirms that IGF-I regulates cyclin D1 expression in a dose-dependent manner. Maximal stimulation was attained at fairly low IGF-I concentration (0.1 ng/ml).

Figure 2.

IGF-I-induces Cyclin D1 Expression in MCF-7 Cells Are Inhibited by PI-3 Kinase Inhibitors, instead of MAPK inhibitor

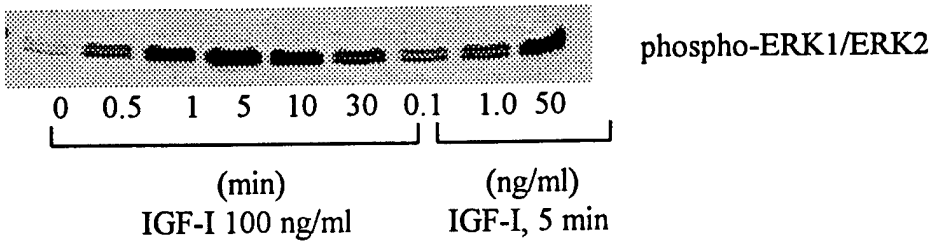
1. synchronize only
2. 10 ng/ml of IGF-I
3. 20 μM of PD98059
4. PD98059 + IGF-I
5. 10 μM of wortmannin
6. wortmannin + IGF-I
7. 100 μM of LY294002
8. LY294002 + IGF-I

Legends:

This result shows the effect of MAPK and PI-3 kinase inhibitors on IGF-I mediated cyclin D1 expression. Our data confirm that PI-3 kinase inhibitors wortmannin and LY294002 down regulated both IGF-I mediated and IGF-I-non-mediated (basal) cyclin D1 expression. In contrast, the MAPK inhibitor PD98059 had no significant effect on either basal or IGF-I mediated cyclin D1 expression.

Figure 3.

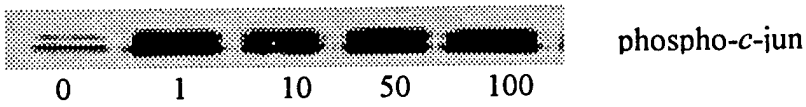
IGF-I Stimulates the Phosphorylation of ERK1/ERK2 in MCF-7 Cells



Legends:

MCF-7 cells were synchronized serum-free M 199 medium over night. The medium was removed and the cultures were washed twice with PBS. IGF-I (100 ng/ml) was added into the culture and incubated as indicated time. In some experiments, various concentrations of IGF-I as indicated above were added into the culture and incubated for 5 min. Cell lysates were collected and the determination of phosphorylation of ERK1/ERK2 in the cell lysates was performed using the method described in the *Materials and Methods*.

IGF-I Stimulates the Activation of SAPK/JNK in MCF-7 Cells



Legends:

MCF- cells were synchronized in serum-free M 199 medium over night. Various concentrations of IGF-I were added into the cultures and incubated for 10 min. Cell lysates were collected and the determination of *c*-jun phosphorylation in the cell lysates was performed using the method described in the *Materials and Methods*.

Fig. 4

Amino Acid Starvation and IGF-I Stimulate the Phosphorylation in MCF-7 Cells

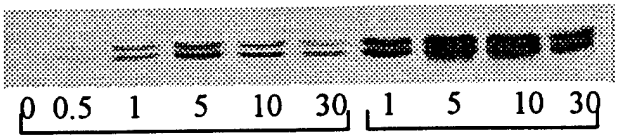
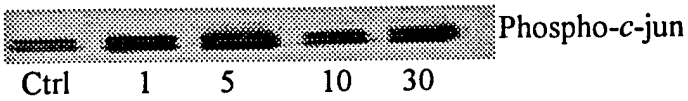


Fig. 5

Amino Acid Starvation Induces c-jun phosphorylation in MCF-7 Cells



2612

TYROSINE PHOSPHORYLATION OF LIPOCORTIN DIMER IN ACTIVATED MICROGLIA. M.Z. Zhang and J.A. McKanna. Vanderbilt Univ. Sch. Med., Nashville, TN 37232

Lipocortin 1 (LC1), a 38-kDa tyrosine kinase substrate with anti-inflammatory and immunosuppressive properties, is constitutively expressed in microglia of the rat CNS. Phosphotyrosine-immunoreactivity (PY-ir) is detected in some LC1+ microglia, and immunohistochemistry demonstrates increased numbers of LC1+ and PY+ microglia after CNS injury. To determine whether glial activation involves tyrosine phosphorylation of LC1 in microglia, immunoprecipitation and western blots were used to compare LC1-PY levels in homogenates of lumbar and cervical spinal cords of rats three days after bilateral sciatic nerve transection. Although LC1-ir and PY-ir each were detected in several discrete bands in the soluble fractions, they coincided only at the 76-kDa band i.e., the LC1 dimer. The presence of LC1-PY was confirmed in anti-PY-sepharose precipitates, where LC1-ir was apparent only at 76-kDa and was increased in the lumbar specimen. These results suggest that tyrosine phosphorylation of LC1 and its dimerization play important roles in microglial activation. Supported by R01NS32660.

2613

TUMOR NECROSIS FACTOR-α IMPAIRS INSULIN STIMULATION OF INSULIN RECEPTOR SUBSTRATE (IRS)-1- AND IRS-2-MEDIATED PHOSPHATIDYLINOSITOL 3-KINASE ACTIVATION.

L. F. del Aguila, K. P. Claffey, and J. P. Kirwan. Noll Laboratory, Penn State University, University Park, PA and *Beth Israel Deaconess Medical Center, Boston, MA.*

It has been suggested that tumor necrosis factor-alpha (TNF-α) plays a critical role in the development of non-insulin-dependent diabetes mellitus (NIDDM). To examine the molecular mechanisms associated with TNF-α and insulin action, we measured the phosphorylation of two mitogen-activated protein kinases (MAPK) known as $p42^{MAPK}$ and $p44^{MAPK}$, insulin receptor substrate (IRS)-1 tyrosine phosphorylation, and IRS-1- and IRS-2-mediated PI3-kinase activation in cultured C_2C_{12} myotubes. We observed that TNF-α repressed insulin-induced $p42^{MAPK}$ and $p44^{MAPK}$ tyrosine phosphorylation. However, pre-treatment with okadaic acid, a phosphatase 2-A (PP-2A) inhibitor, prevented TNF-α inhibition of insulin-induced $p42^{MAPK}$ and $p44^{MAPK}$ tyrosine phosphorylation, suggesting a phosphatase-dependent mechanism in the inhibition of MAPK by TNF-α. In addition, insulin-stimulated IRS-1 tyrosine phosphorylation was impaired in cultured cells pre-treated with TNF-α. Furthermore, insulin stimulation of IRS-1- and IRS-2-mediated PI3-kinase activation were diminished by TNF-α. Unlike the effects on $p42^{MAPK}$ and $p44^{MAPK}$, okadaic acid did not prevent TNF-α inhibition of insulin-induced PI3-kinase activation, suggesting a PP-2A-independent mechanism in the inhibition by TNF-α of insulin-induced PI3-kinase activation. These data indicate that TNF-α may contribute to the development of insulin resistance in skeletal muscle by impairing tyrosine phosphorylation on $p42^{MAPK}$ and $p44^{MAPK}$ as well as IRS-1- and IRS-2-mediated PI3-kinase activation. *Supported by NIH grant R29 AG12834-03*

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EXPRESSION OF THE FLT3 RECEPTOR AND RESPONSES TO THE FLT3 LIGAND BY HUMAN DERMAL MICROVASCULAR ENDOTHELIAL CELLS. H. Chen, X. Zhang, and M. Karasek, Dept. of Dermatology, Stanford Univ. Sch. of Med. Stanford, CA. 94305

Considering the close relationships and similarities between hematopoietic progenitor cells and endothelial cells, we analyzed the expression of the fms-like tyrosine kinase receptor 3 (flt3R), its ligand, (flt3L), and the responses to exogenous flt3 in human dermal microvascular endothelial cells (HDMEC). The expression of flt3R and flt3L was detectable at both the mRNA and protein levels in isolated HDMEC by RT-PCR, immunofluorescence staining, FACS, and Western blotting. A 1,391 bp segment of genomic DNA from HDMEC, which corresponds to exon 10 of the transmembrane domain and its flanking region, has been cloned and sequenced. An inverted Alu-Sx -like sequence with the direct repeats of GCTGGGATTACAGGC was identified in intron 9. Both IL-1 and PMA upregulated flt3R and flt3L. No significant effects could be detected when HDMEC were treated with flt3L, TNF-*a*, or TGF-*b* alone. However, treatment with flt3L combined with bFGF synergistically increased the proliferation of HDMEC. In contrast, when HDMEC were treated with flt3L and TNF-*a* or TGF-*b*, apoptosis was activated and proliferation was inhibited. These results demonstrate an unusual and novel interaction of cytokines and growth factors in the response of HDMEC to flt3.

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CLONING AND EXPRESSION PROFILING OF MURINE clk2 mRNA. Matthew C. Weber and Mark L. Tykocinski, Department of Pathology, Case Western Reserve University, Cleveland, OH 44106.

The clk (cdc2-like kinase) family of dual-specificity protein kinases are capable of phosphorylating both serine/threonine and tyrosine residues. They are thought to be involved in growth control and development, possibly by regulating mRNA splicing. In the case of clk2, only the human cDNA has been cloned to date, and even here, its expression profile was limited to leukocytes. In the course of screening a murine cDNA expression library made from mouse decidual endometrium at day 7-8 of gestation, we identified a 1.5 kb cDNA clone with high sequence homology to the human clk2 cDNA. When this clone was used as a probe in Northern blot analyses, a 2.2 kb message was detected in day 7 (highest expression), 12, 14, and 18 placental and decidual endometrial tissues, but not in normal uterus. Spleen, brain, and whole embryo were also positive, but interestingly, the highest expression was observed in a murine megakaryocytic cell line, L8057. These findings set the stage for targeted murine clk2 functional analyses.

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THE SIGNAL TRANSDUCTION PATHWAYS IN BREAST CANCER CELL MCF-7 UPON AMINO ACID STARVATION INVOLVED IN MAPK, AND SAPK/JNK PATHWAYS. (JV. Vadgama), W. Liu, and Y. Wu. Charles R. Drew University of Medicine and Science, Department of Medicine, Los Angeles, CA 90059.

We have tested the hypothesis that signal transduction pathways in breast cancer involve activation of MAPK, ERK1/ERK2, and SAPK/JNK pathways. These signals cause activation of oncogenes and increase in cell proliferation. Amino acid starvation is a physiological condition that results in protein deprivation resulting from cancer cachexia. We have examined the effect of AA starvation on the specific signal pathways. Our results demonstrated an increase in phosphorylation of ERK1/ERK2 in response to time-dependent AA starvation, with peak activity at five minutes. In addition, we confirmed using Western blot analysis, the activation of tyrosine kinase phosphorylation within one minute of AA starvation. Stress-activated protein kinase, SAPK/JNK were also induced in response to AA starvation, resulting in c-jun phosphorylation. c-fos activation is a down-stream event resulting from phosphorylation of ERK1/ERK2. Our studies confirmed that AA starvation caused an upregulation of c-fos mRNA. In summary, protein kinases such as ERK1/ERK2 and SAPK/JNK play an important role in response to AA starvation in cancer cells.

Supported by NIH, NCRR-G12RR03026 and NCI-P30CA49095-09.

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THE INVOLVEMENT OF PROTEIN TYROSINE KINASE (PTK) IN OSMOREGULATION OF Na^+ TRANSPORT IN RENAL EPITHELIAL A6 CELLS. Naomi Niisato, Mingyao Liu, Willy Van Driessche, and Yoshinori Marunaka, Hospital for Sick Children and Toronto Hospital, University of Toronto, Toronto, Ontario, Canada and K. U. Leuven, Belgium

We have shown that hyposmolality in the extracellular solution stimulates Na^+ transport in renal epithelial A6 cells. The mechanisms by which the change in extracellular osmolality initiates activation of a signal transduction pathway and how the pathway results in activation of Na^+ transport are still unknown. We investigated the regulatory mechanism of Na^+ transport stimulated by hypotonic stress in A6 cells. To study a possible role of PTK for hyposmolality-mediated signaling, effects of PTK inhibitors on the hyposmolality-stimulated Na^+ transport were examined. PTK inhibitors markedly abolished the hyposmolality-stimulated amiloride-sensitive short-circuit current (ami-Isc). Hyposmolality caused time-dependent activation of PTK, leading to an increase in tyrosine phosphorylation in multiple proteins which was diminished by pretreatment with PTK inhibitors. Pretreatment with brefeldin A, which blocks translocation of proteins, abolished the hyposmolality-induced increase in ami-Isc. Single channel recording data show that hyposmolality dramatically increased the number of amiloride-blockable Na^+ channels in the apical membrane. PTK inhibitor markedly abolished the hyposmolality-induced increase in number of the Na^+ channel, suggesting PTK is involved in the process of the hyposmolality-stimulated translocation of Na^+ channels to the apical membrane. Hyposmolality also increased membrane capacitance which was blocked by pretreatment with PTK inhibitors. These results suggest that hypotonic stress stimulates to translocate Na^+ channels to the apical membrane via PTK activation, resulting in the increase in amiloride-sensitive Na^+ transport in A6 cells. Supported by KFC, MRC & OTS. ML and YM are scholars of MRC.

rearrangements were not observed. Instead one of four pathways of gene activation could be identified with stepwise selections: (1) Early amplification and overexpression of the amplified allele; increased amplification and expression with advancing drug selection. (2) Early overexpression of a single allele; amplification of the overexpressed allele occurred only at high levels of expression (3) Overexpression of both alleles mediated by trans-acting factors as evidenced by increased activity of an *MDR-1* reporter construct, and increased expression of the transcription factor, YB-1; amplification of a single allele only at high levels of resistance. (4) Overexpression of both alleles early in selection, followed by overexpression of a single allele and consequent amplification of the over-expressed allele. We conclude that (1) activation of a silent *MDR-1* gene requires a major change: gene rearrangement with capture of *MDR-1*, and (2) increased expression of a constitutively expressed *MDR-1* gene results from transcription factor overexpression or gene amplification. Once amplification occurs, increased expression of transcription factors does not appear necessary.

#3114 Co-expression and co-amplification of sequences upstream of the *MDR-1* gene in unselected and drug selected human cell lines. Wang, Z., Mickley, L.A. Lee, J.-S., Greene, E. and Fojo, T. *Medicine Branch, NCI, and NCHGR, NIH, Bethesda, MD and Department of Internal Medicine, Gyeongsang National University, Kyungnam, South Korea.*

In previous studies sequences upstream of *MDR-1* in KB C-1 RNA were thought to arise from an upstream promoter. Using 5' RACE cloning we isolated an additional 157 bp proximal to the previously reported 94 bp. YAC clones localized these 251 base pairs 100–300 kb upstream (telomeric) of *MDR-1*. PCR expression of the 251 base pairs was examined in unselected and drug selected cell lines. In 32 unselected cell lines (27 *MDR-1* negative and 5 *MDR-1* positive) expression was detected only in *MDR-1* negative ZR-75B cells. In contrast expression was detected in 14/28 drug selected cell lines; including 14/18 with amplification of *MDR-1*. In the drug selected cell lines, expression of the 251 bp was independent of *MDR-1* (6/14) or was part of the *MDR-1* transcript (8/14). Using RNA from KB V-1 cells and the 251 bp as a probe, two fragments were detected: a 4.5 kb *MDR-1* mRNA and an additional 1.0 kb fragment. Multiple attempts to isolate the 1.0 kb transcript from sized cDNA libraries were unsuccessful. These data suggest there's a region with promoter activity located 100–300 kb 5' of the *MDR-1* start site. Expression of 251 bp from this region is seen in drug selected cell lines and coincides with amplification of this region. While *MDR-1* transcripts can begin in this upstream region, this region can also be transcribed independent of *MDR-1*. The present study suggests the promoter activity in this region may not be specific for *MDR-1*.

#3115 5' CpG island demethylation and chromatin structure are associated with transcriptional activation of human multidrug resistance 1 (MDR1) gene in human cancer cells and hematopoietic neoplasia. Kusaba, H., Nakayama, M., Nagayama, J., Kohno, K., Komatsu, H., Ueda, R., Kozuru, M., Uchiumi, T., Kuwano, M., and Wada, M. *Kyushu University, Fukuoka 812-82, Japan, University of Occupational and Environmental Health, Kitakyushu 807, Japan, Nagoya City Univ. Sch. Med., Nagoya. National Kyushu Cancer Center, Fukuoka, Japan.*

Selection of mammalian cells for resistance to vincristine often induces overexpression of multidrug resistance1 gene (MDR1) which encodes the P-glycoprotein (P-gp), as a result of gene amplification or transcriptional activation. However, the precise mechanism underlying such transcriptional activation of MDR1 remains unclear. In this study, the relation between methylation status in the MDR1 promoter region and transcriptional activation of MDR1 has been investigated. We examined P-gp overexpressing cancer cell lines. Determination of methylation status at MspI HpaII sites revealed hypomethylated in P-gp-overexpressing cell lines, and hypermethylated in the parental cells. Prior treatment with DNA methyltransferase inhibitor resulted in a 10-fold increase in the frequency of vincristine-resistant clones generated by subsequent exposure of the cells to vincristine, suggesting inverse correlation of methylation status and MDR1 overexpression. Although the binding of YB-1, a transcription factor implicated in MDR1 expression, appeared not to be altered directly by hypomethylation. DNaseI hypersensitive site of the MDR1 promoter region were differently observed between the P-gp overexpressing cells and the parental cells, suggesting altered chromatin structure of the relevant region in the multidrug resistant cells. We examined methylation status and MDR1 expression in acute myelogeneous leukemia, and also observed inverse correlation in clinical samples. Methylation status of the MDR1 promoter is expected to play a key role in MDR1 overexpression in cancer cells.

#3116 Isolation and characterization of full-length and alternatively spliced *MDR1* transcripts in multidrug-resistant human cancer cell lines using long RT-PCR. Hu, Y., Tanzer, L. R., Cao, J., Geringer, C. D., and Moore, R. E. *Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285-0424*

Recent advances in RT-PCR technology allow the full-length coding regions of even large mRNAs to be copied in one step in one tube. With stringent long RT-PCR conditions, we can further insure that smaller than full-length PCR products correspond to true splice variant mRNAs rather than being technical artifacts. To better understand the full range of *MDR1* gene expression in human leukemia cell lines, we have analyzed splice variant production in a systematic manner utilizing long RT-PCR technology. Twenty different *MDR1* cDNAs were cloned and sequenced. Two of these clones coded for full-length, wild-type *MDR1*, the rest encoded splice variants. Fourteen of the less than full-length clones were identical and represented a very prominent splice variant mRNA that can be detected in two drug resistance tumor cell lines (HL60/VCR and U937-10A). This splice variant is derived from the splicing of exons 6 to 18 from the *MDR1* mRNA. It is predicted this one mRNA would encode two proteins. The larger *MDR1* protein would contain transmembrane domains 9 to 12 and one ATP binding domain. In support of this model, *in vitro* transcription and translation studies indicate that this *MDR1* splice variant mRNA does encode both predicted proteins. The biological significance of this novel mRNA is being further investigated.

#3117 Fine mapping of a chromosome 7 abnormality associated with a multidrug resistant phenotype. de Silva, M.G., Kantharidis, P., Scherer, S.W., Rayeroux, K., Campbell, L., Tsui L.-C., Zalcberg, J.R. *Division of Haematology & Medical Oncology, Peter MacCallum Cancer Institute, Melbourne, Australia, Department of Genetics, The Hospital for Sick Children, Toronto, Canada, Department of Cytogenetics, St. Vincent's Hospital, Melbourne, Australia.*

Both the expression of the multidrug transporter, P-glycoprotein (Pgp) and abnormalities of the long arm of chromosome 7 have been shown to be adverse prognostic indicators in acute leukemias. In this study the breakpoints of a clonal duplication, dup(7)(q11.1q31.1), inherited with the classical multidrug resistant phenotype in a drug-resistant derivative of a human T-cell leukemia cell line were characterized. The position of the duplication was of interest as the gene which encodes Pgp, *mdr1*, is located on the long arm of chromosome 7 at position 7q21.1. With a panel of well characterized YAC clones the duplicated segment was found to be a direct tandem duplication somewhat larger than estimated by conventional cytogenetics. The proximal and distal breakpoints of the abnormality were located and a YAC clone spanning the distal breakpoint was identified. This clone is of particular interest as it harbors the markers D7S523 and D7S471 close to which a putative tumor suppressor gene is thought to lie. Further examination of the breakpoint region is ongoing and may illuminate the mechanism of Pgp upregulation as well as providing information about a tumor suppressor gene.

CELL AND TUMOR BIOLOGY 19: Signaling through MAP Kinases

#3118 Stimulation of DNA synthesis in epithelial cells by multiple agonists via a SEK/JNK/c-Jun dependent mechanism. Kelly L. Auer, Martin McMahon, Michael Birrer, and Paul Dent. *Department of Radiation Oncology, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298-0058.*

The ability of signaling via both the Raf/MEK/MAPK and SEK/JNK/c-Jun cascades to stimulate or inhibit DNA synthesis in primary cultures of adult rat hepatocytes was examined. Treatment of hepatocytes with media containing hyperosmotic glucose (75 mM final), Tumor Necrosis Factor α (TNFα, 1 ng/ml final), and Hepatocyte Growth Factor (HGF, 1 ng/ml final) caused activation of the stress activated protein kinase (SAPK) JNK1 (c-Jun NH_2-terminal kinase). Glucose, TNFα, or HGF treatments increased phosphorylation of c-Jun at Serine 63 in the transactivation domain, and stimulated DNA synthesis. Infection of hepatocytes with poly-L-lysine coated adenoviruses coupled to constructs to express either dominant negative Ras ^{N17}, dominant negative Rac1 ^{N17}, dominant negative Cdc42 ^{N17}, dominant negative SEK1, or dominant negative JNK1 blunted the abilities of glucose, TNFα, or HGF to increase JNK1 activity, to increase phosphorylation of c-Jun at Serine 63, and to stimulate DNA synthesis. Furthermore, infection of hepatocytes by a recombinant adenovirus to express dominant negative c-Jun (TAM67) also blunted the abilities of glucose, TNFα, and HGF to stimulate DNA synthesis. In contrast, when MAP kinase was chronically activated in hepatocytes, via infection of hepatocytes with a poly-L-lysine coated adenovirus coupled to a construct to express an inducible estrogen receptor-Raf-1 fusion protein, expression of p21 $^{Cip-1/WAF1}$ and p16 ^{INK4a} cdk inhibitor proteins increased and DNA synthesis decreased. This data demonstrates that the key mechanism by which agonists stimulate DNA synthesis in hepatocytes is via the SEK/JNK/c-Jun pathway.

#3119 IGF-I induced mitogenesis of breast cancer cells involves MAPK, and the PI-3 kinase pathways. Liu, W., Chillar, R., and Vadgama, J.V. *Department of Medicine, Charles R. Drew University of Medicine and Science, Los Angeles, CA 90059.*

The insulin-like growth factors (IGFs) stimulate cell proliferation and differentiation. Cumulative evidence have shown that IGF-I plays an important role in the mitogenesis of breast cancer cells. IGF-I stimulates cell division by modulating the events occurring during the early G1 phase, but little is known about the signal transduction pathway involved in these events. In this study, we examined the signal transduction pathway involved in the IGF-I-induced cyclin D1 expression in human breast cancer cell MCF-7. Mitogen-activated protein kinases, ERK1/ERK2

and c-jun in MCF-7 cells are rapidly activated and phosphorylated in response to the addition of IGF-I. IGF-I stimulates cyclin D1 expression in MCF-7 cells in a time/dose-dependent manner. This stimulation can be blocked by two unrelated PI-3 kinase inhibitors, Wortmannin and LY294002, instead of MAP kinase inhibitor. In addition, IGF-I strongly stimulates cyclin D1 mRNA expression in MCF-7 cells and this stimulation can also be blocked by PI-3 kinase inhibitors, indicating that the regulation of IGF-I-induced cyclin D1 expression in MCF-7 cells occurs at transcription level. These results indicate that IGF-I-induced mitogenesis in MCF-7 cells are mediated by PI-3 kinase signaling pathway, in addition to the MAPK pathway.

#3120 Ras stimulates DNA topoisomerase IIα through MEK: A link between oncogenic signaling and a therapeutic target. Guan Chen[1], Dennis Templeton[2], D. Parker Suttle[3], and Dennis W. Stacey[1]. *1) Department of Molecular Biology, The Cleveland Clinic Foundation; 2) Institute of Pathology, Case Western Reserve University, Cleveland, OH; 3) Department of Pharmacology, The University of Tennesse, Memphis, TN.*

Topoisomerase IIa (topo IIa), a nuclear protein required for maintenance of DNA topology, and a target of anti-tumor treatments, was elevated in *ras*-, *src*- and *raf*-transformed NIH3T3 cells. Anti-Ras antibody decreased topo IIa levels in *ras*- and *src*- but not in *raf*-transformed cells, while microinjection of oncogenic Ras protein increased topo IIa protein in non-transformed NIH 3T3 cells. Further studies demonstrated stimulation of topo IIa promoter activity by oncogenic Ras, which occurs independently of cell cycle progression, since similar effects were observed in PC12 cells where Ras is not mitogenic. Transactivation of topo IIa required MEK/ERK, since either a dominant inhibitory MEK construct or a MEK-specific chemical inhibitor completely abolished ERK activation together with topo IIa promoter stimulation by Ras. Moreover, stress-associated protein kinase (SAPK) was also involved in Ras-induced topo IIa transactivation, as a dominant inhibitory SAPK kinase (SEK/AL) also reduced Ras-induced topo IIa promoter activity. As a direct confirmation that ERK and SAPK are involved in topo IIa promoter activation, these two kinases and the promoter activity were stimulated independently of Ras activity by the action of a constitutively active MEKK. Thus, both oncogenic and stress related signaling are able to stimulate the topo IIa promoter utilizing pathways which independently induce ERK and SAPK activity. Taken together, these studies demonstrated the direct role of Ras signaling in stimulation of topo IIa expression, and thereby establish a link between the action of a common tumor mutation and the target of anti-tumor treatments.

#3121 TGFβ signaling components and cross-talk between the Smad and Ras/Mapk signaling cascades in epithelial cells. Frey, R.S., Yue, J., Zhu, Q., Fletcher, T.M., Liu, X., and Mulder, K.M. *Penn State University College of Medicine, Hershey, PA 17033, USA.*

We previously demonstrated that Ras activation was necessary and sufficient for TGFβ-mediated Erk activation and up-regulation of $p21^{Cip1}$ and $p27^{Kip1}$; it was partially required for TGFβ-mediated inhibition of Cdk 2 activity, cyclin A expression, and DNA synthesis. In addition, TGFβ activated Erk2 and Sapk/JNK in human breast cancer cells (BCCs); these effects were sustained and associated with inhibition of DNA synthesis. Here we transiently transfected intestinal epithelial cells (IECs) and BCCs with a rat homologue of the drosophila *mad* gene (termed *Rsmad1*), which was cloned from an IEC cDNA library. Both TGFβ and its superfamily member BMP resulted in a rapid phosphorylation of RSmad1 *in vivo*, using either an antibody against the epitope-tagged RSmad1 being expressed or a Smad1-specific polyclonal antiserum. Pre-treatment of the cells with the specific MEK inhibitor PD98059 resulted in a partial inhibition of RSmad1 phosphorylation by TGFβ or BMP. This occurred under conditions in which Erk phosphorylation by both growth factors was blocked. In addition, dominant-negative MEK resulted in a partial reversal of the ability of TGFβ or BMP to inhibit DNA synthesis. Collectively, our results suggest that MEK may play an important role in both TGFβ and Smad signaling in epithelial cells. This work was supported by NIH grants CA51452, CA54816, CA68444, and CA59552 to KMM.

#3122 Reduced glutathione (GSH) as a major determinant of redox signaling in H4 rat hepatoma cells. Yurkow, E.J. *Dept. of Pharmacology & Toxicology, Rutgers Univ., Piscataway, NJ 08855.*

A pro-reducing cellular redox status is thought to inhibit normal apoptotic and growth-regulatory processes by uncoupling specific redox-sensitive signaling events. In the current study, the influence of GSH levels on redox-sensitive signaling was investigated in control and oxidant-treated H4 rat hepatoma cells. Cellular GSH and peroxide levels were determined flow-cytometrically. Treatment of cells with hexavalent chromium (Cr(VI)) induced the production of cellular peroxides which was correlated with the activation of the mitogen-activated protein kinases (MAPK) ERK1 & ERK2. These effects were observed with minimal alterations in the steady-state levels of GSH. Increasing GSH levels 5-fold using the GSH precursor, N-acetyl-L-cysteine, decreased both Cr(VI)-induced peroxide generation and MAPK activation. Conversely, depleting GSH levels by 50% using the GSH synthesis inhibitor buthionine sulfoximine, increased the sensitivity of the cells to Cr(VI). Interestingly, depleting cellular GSH levels further (to 30% of control) dramatically attenuated Cr(VI)-induced MAPK activation. The inability to activate MAPK by Cr(VI) in these cells was specific for oxidants, since these kinases could be maximally activated using other agents (i.e., phorbol esters). Our results suggest that cellular GSH levels define the redox signaling potential of cells. In some circumstances, GSH acts as a reducing agent that can uncouple redox-dependent signaling events from normal growth/apoptosis regulatory processes. In other situations, GSH acts as an essential requirement for redox-sensitive signaling.

#3123 Balance between activated-STAT and MAP kinase regulates the growth of human bladder cell lines after treatment with EGF. Kawamata, H., Hattori, K., Tamatani, T., Hino, S., Uchida, D., Sato, M., and Oyasu, R. *Second Dept of Oral and Maxillofacial Surg., Tokushima Univ. Sch. of Dent., Tokushima 770 Japan, and Dept. of Pathol., Northwestern Univ. Med. Sch, Chicago, Illinois 60611.*

Epidermal growth factor (EGF) is a potent mitogen, and its action is mediated by the mitogen-activated protein kinase (MAPK) in several cell types. Recently, it is reported that EGF activates the signal transducers and activators of transcription (STAT) 1 and STAT3, induces the expression of p21 waf1, and subsequently inhibits the growth of several cancer cells. In this study, we used human carcinoma cell lines (A431, T24 and RT4) and immortalized nontumorigenic human urothelial cell lines (1T-1, 1T-2, and 1T-3). Their anchorage-dependent growth after EGF (10–100 ng/ml) treatment was compared with the activities of MAPK and STAT, which were determined by Western blotting and mobility shift assay using a p21 waf1 promoter sequence, respectively. The induction of p21 waf1 mRNA was also examined. EGF receptor was expressed in all cell lines (7×10^4 to 3×10^5 per cell). EGF inhibited the growth of A431 (epidermal cancer cells) and T24 (bladder cancer cells), but did not affect the growth of RT4 (bladder cancer cells). EGF stimulated the growth of 1T-1 strongly, and 1T-3 slightly, but not all of 1T-2. EGF activated the MAPK strongly in 1T-1 and A431, and slightly in RT4, but marginally in 1T-2, 1T-3 and T24. Furthermore, we detected the activation of STAT1 in A431 and 1T-3, and STAT1 and STAT3 in T24 after EGF treatment. EGF also enhanced the expression of p21 waf1 mRNA in A431, T24, 1T-2, and 1T-3. These results suggest that balance between the STAT-induced p21 waf1 and MAPK activities regulates the growth of human bladder cell lines after EGF treatment. (Supported by CA14649.)

#3124 MAP kinase is activated by 12(S)HETE. Karoly Szekeres and Kenneth V. Honn. *Wayne State University, Departments of Radiation Oncology (KS; KVH), Pathology (KVH) and Chemistry (KVH) Detroit, MI, 48202.*

12(S)HETE is a 12 Lipoxygenase product of arachidonic acid, which modulates several steps of tumor metastasis, i.e. it enhances cell adhesion to endothelial cells and basal membrane, increase protease secretion and prevents apoptosis. We have previously shown, that some of the 12(S)HETE effects are mediated by specific subtypes of protein kinase C (PKC). We also have demonstrated tyrosine phosphorylation of 42/44 kDa proteins after 12(S)HETE treatment. In A431 carcinoma cells we have identified these bands as the 42/44 kDA mitogen activated protein kinases (MAP kinase). By Western blotting, immunoprecipitation and in vitro kinase assay we demonstrated that this activation is time and concentration dependent with a maximum at 10 minutes and 100 nM concentration of 12(S)HETE. Using specific inhibitors of the classical forms of PKC (Calphostin C) and phosphatidyl inositol 3 kinase (P13K) (Wortmannin and LY294002) we have demonstrated, that MAP kinase activation is dependent on both PKC and P13K. MAP kinases are involved in mediating a wide array of biological behaviour, most importantly cellular proliferation. Showing the activation of these kinases in a system, where 12 Lipoxygenase is expressed opens up potential new mechanisms of action of 12(S)HETE and possibly the discovery, that previously unknown cellular behaviors are regulated by this bioactive lipid.

#3125 Functional expression of bombesin receptor in human glioblastoma cell lines and its role in mitogenesis. Sharif TR, Luo W, and Sharif M. *Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis, TN 38105.*

Bombesin and bombesin-like peptides [gastrin-releasing peptide (GRP) and neuromedin B (NMB) peptide] regulate biological responses through the activation of two distinct receptor subtypes (BB_1 and BB_2). Bombesin and bombesin-like peptides and their respective receptors have been shown to play a role in stimulating the growth of human tumor-derived cell lines such as those derived from small cell lung carcinoma. In this study, we examined the functional expression and the role of bombesin receptor in stimulating the growth of glioblastoma cells. Functional bombesin receptors were present in 85% of human glioblastoma cell lines examined. Bombesin stimulated the release of intracellular Ca^{2+} in nine adult and two pediatric glioblastoma cell lines. Stimulation of U-373MG cells with bombesin or GRP induced DNA synthesis and stimulated the phosphorylation of the mitogen-activated protein kinases (MAPKs). Bombesin and GRP showed similar potencies in stimulation of intracellular Ca^{2+} release and activation of the MAPK phosphorylation in U-373MG cells, whereas NMB was less potent. A bombesin receptor antagonist blocked bombesin induced Ca^{2+} release and attenuated MAPK phosphorylation in U-373MG cells demonstrating that bombesin is acting through a receptor-dependent mechanism. In conclusion, this study demonstrates the functional expression of bombesin receptor in human glioblastoma cell lines and attempts to characterize mitogenic signaling induced by this receptor in U-373MG glioblastoma cells. (Supported by ALSAC and CA71756).